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PRINCIPAL INVESTIGATOR: Simona Parrinello
Judith Campisi, Ph.D.

CONTRACTING ORGANIZATION: Ernest Orlando Lawrence Berkeley
National Laboratory
Berkeley, California 94720

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Simona Parrinello
Judith Campisi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Ernest Orlando Lawrence Berkeley National Laboratory
Berkeley, California 94720

E-Mail: sparrinello@lbl.gov and pmgale@lbl.gov

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Cancer incidence rises exponentially with age. We have previously shown that human senescent cells stimulate the proliferation of premalignant and malignant breast epithelial cells in culture. Senescent cells exist and accumulate with age in *vivo*, suggesting that senescence might contribute to the increase of breast cancer with age. This project test the hypothesis that senescent mouse fibroblasts also stimulate epithelial growth by creating a permissive environment for the expression of epithelial malignancies.

Here we report the development of a co-culture system for studying the effect of presenecent and senescent mouse fibroblasts on mouse mammary epithelial cells. Using this system, we show that senescent mouse fibroblasts senescence is induced by oxidative stress and differs from human fibroblasts senescence. To establish more physiological conditions for their growth, we cultured mouse fibroblasts in low oxygen and induced cellular senescence with sublethal doses of X-irradiation. Future experiments will be aimed to test whether under these conditions mouse fibroblasts stimulate epithelial proliferation similarly to human cells.

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Table of Contents

Introduction

In humans, the incidence of breast cancer rises exponentially with age[1,2]. We proposed that senescent cells, which accumulate with age *in vivo*[3-5], might contribute to this rise by altering the microenvironment of and creating a procarcinogenic milieu for breast epithelial cells. In support of this hypothesis, our group showed that senescent human fibroblasts stimulate the growth of premalignant and malignant epithelial cells in culture and in immunocompromised mice[6]. To further understand the role of senescent cells in cancer *in vivo*, we extended these studies to the mouse, the most widely used model system for breast cancer. Specifically, our goal is to test whether senescent mouse fibroblasts promote carcinogenesis similarly to human fibroblasts, identify any differences between mouse and human senescence, and establish conditions under which the senescent phenotype of mouse fibroblasts most closely mimics that of human fibroblasts. Therefore, as proposed, we developed culture systems to study the effect of senescent mouse embryo fibroblasts (MEFs) on mouse mammary epithelial cell proliferation and have begun a detailed characterization of MEF senescence. These experiments will lay the basis for the development of an organismal model for senescence- and age-related cancer.

Body

Replicatively senescent mouse fibroblasts might, similarly to senescent human fibroblasts, enhance the growth and tumorigenic potential of epithelial breast cells. This effect might be due to the ability of senescent mouse fibroblasts to stimulate the proliferation and/or disrupt the differentiation of normal or premalignant epithelial cells.

In the past year, I have successfully established experimental conditions to test these hypotheses in culture and the results thus far obtained are described below.

1. *Establishment of fibroblastic cultures.* I have isolated primary embryonic (MEFs) and adult mammary fibroblasts from Balb/c animals and established presenescent and senescent cultures. All these experiments were performed under standard culture conditions, including 20% O_2 tension. MEFs senesced after 10-15 population doublings (PDs), as reported[7], whereas mammary cells underwent 3-6 PDs, as expected from their more advanced developmental stage, before reaching a senescent state. A representative growth curve for MEFs is shown in fig. 1a. In both cases, the senescent cultures were morphologically homogenous, consisting of large, flat cells (fig. 1b and 1c) and did not show any increase in cell number over a period of 7-14 days.

2. *Isolation of primary mammary epithelial cells.* I also isolated primary mammary epithelial cells (MECs), initially as organoids, from the glands of virgin Balb/c females. The cells were grown on uncoated tissue culture plastic, or tissue culture dishes containing collagen or Matrigel (a lamin-rich mixture of extracellular matrix components), to characterize their growth and differentiation properties. The cells could be successfully grown on plastic as single cell-monolayers for 2-5 days. Epithelial morphology was better preserved when the organoids were plated in collagen gels[8]. Under these conditions, the cells remained associated as ductal-like structures (fig. 2a) and could be maintained for up to 10 days[8]. Growth in Matrigel produced alveolar structures with differentiated morphology: correct cell polarity, lumen formation and deposition of a basement membrane (fig. 2b). These results indicate that primary cells can be cultured and induced to differentiate successfully.

3. *Establishment of SCp2 cells cultures.* SCp2 cells are premalignant mammary epithelial cells, which I cultured and induced to differentiate as reported[9]. In the presence of lactogenic hormones and a basement membrane, the cells underwent morphological differentiation similarly to primary cells (see fig. 2b). Differentiation was also obtained in collagen gels. However, the alveoli seemed to be less stable under these conditions and therefore Matrigel will be used for all future experiments.

4. *Co-culture of mouse mammary cells on fibroblast monolayers.* Presenescent ($5x10^4$) and senescent ($1x10^5$) MEFs were allowed to attach to 6-well culture dishes overnight, and incubated in complete medium for 1-2 d to generate lawns

with similar numbers of cells. Mouse epithelial cells, primary or SCp2, were then seeded ($2x10^4$/well) on the fibroblast lawns in 2 ml of growth factor-deficient medium. Eight days later, the cultures were fixed in methanol, stained with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) and photographed. Five images per well were then used to quantify epithelial growth using the NIH-image software. As shown in figure 3, no difference could be detected in the growth of either primary or SCp2 cells in the presence of presenescent or senescent MEFs. A total of 3 experiments were performed per condition.

Our preliminary results had suggested that senescence in standard culture is an oxidative stress response and that differences exist between human and mouse fibroblasts at senescence. My data described above are consistent with those results. Given the clear lack of a stimulatory phenotype by replicatively senescent mouse fibroblasts, we have decided it would not be a prudent use of time to extend the results to the fully malignant cell line JC, as originally proposed in the Statement of work. Instead, as proposed, we started characterizing the phenotype of senescent Balb/c fibroblasts obtained under more physiological conditions, where we expect senescent MEFs to promote epithelial growth. To this end, I have initiated the following experiments.

1. *Culture of Balb/c MEFs in low oxygen.* Balb/c MEFs were explanted and grown in 3% O_2 and their lifespan compared to that of parallel cultures in 20% O_2. The average of 5 experiments is shown in fig.4. In low oxygen, Balb/c MEFs undergo a 3-fold life-span extension after which they arrest growth with a senescent-like morphology.

2. *Irradiation of MEFs.* Sublethal doses of X-ray are known to induce a senescent-like phenotype in human fibroblasts[10]. To determine the optimal dose of X-ray for induction of senescence in MEFs, I irradiated 3 parallel cultures grown in 3% O_2 with 3, 5 and 7 Gy. The highest dose seemed to induce the strongest and most reproducible phenotype. After treatment with 7 Gy, MEFs remained viable, arrested proliferation almost completely and acquired a senescent morphology. The dose of 7 Gy will therefore be used for all future experiments.

Key Research Accomplishments

- Presenescent and senescent mouse fibroblast cultures have been established under standard conditions.
- Primary mammary epithelial and SCp2 cells have been successfully cultured and induced to differentiate.
- Replicative senescent mouse fibroblasts obtained in 20% O2 do not stimulate the growth of primary or premalignant mammary epithelial cells in co-culture.
- Balb/c MEFs grown in 3% O_2 undergo a dramatic life-span extension after which they senesce.
- Irradiation of presenescent MEFs with 7 Gy induces a strong and stable senescent arrest without compromising cell viability.

Conclusions

In summary, we developed a culture system to test the effect of senescent mouse fibroblasts on the proliferation of mammary epithelial cells. Using this system, we found no difference in epithelial growth stimulation between presenescent and replicatively senescent fibroblasts derived under standard conditions. These data are consistent with previous preliminary work that had shown differences between human and mouse cells at senescence in 20% oxygen. Such differences are most likely related to the higher sensitivity of murine cells to oxidative stress. Therefore, we started testing more physiological conditions, including 3% O_2, for the growth of mouse fibroblasts. We have shown that in low oxygen MEFs still undergo a senescent arrest and that cellular senescence can also be induced by X-ray treatment. Our future work will be devoted to testing whether senescent mouse cells obtained under these conditions stimulate epithelial growth.

References

1. Balducci L, Beghe' C. 2001. Cancer and age in the USA. *Critical Reviews in Oncology/Hematology*. 37:137-145.
2. Hanahan D.,Weinberg R. A. 2000. The hallmarks of cancer. *Cell* 100:57-70.
3. Dimri G. P., Lee X., Basile G., Acosta M., Scott G., Roskelley C., Medrano E. E., Linskens M., Rubelj I., Pereira-Smith O., Peacock M. and Campisi J. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A*. 92:9363-9367.

4. Pendergrass W. R., Lane M. A., Bodkin N. L., Hansen B. C., Ingram D. K., Roth G. S., Yi L., Bin H., Wolf N. S. 1999. Cellular proliferation potential during aging and caloric restriction in rhesus monkeys (Macaca mulatta). *J Cell Physiol.* 80:123-30.

5. Mishima K., Handa J. T., Aotaki-Keen A., Lutty G. A., Morse L. S. and Hjelmeland L. M. 1999. Senescence-associated beta-galactosidase histochemistry for the primate eye. *Invest Ophthalmol Vis Sci.* 40:1590-1593.

6. Krtolica A., Parrinello S., Lockett S., Desprez P. Y. and Campisi, J. 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A.* 98:12072-12077.

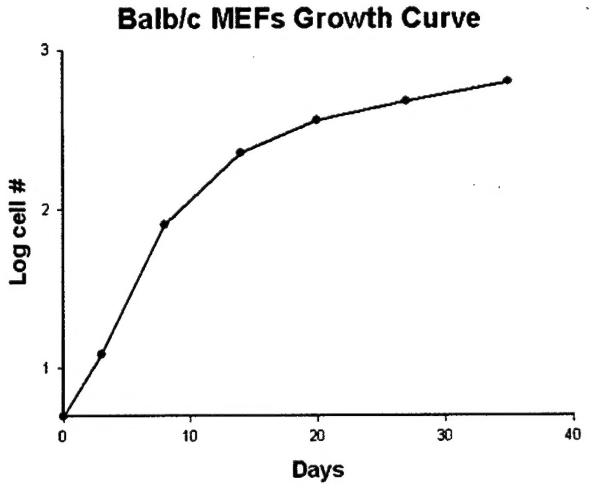
7. Todaro, G. J. & Green, H. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established cell lines. *J. Cell Biol.* **17**, 299-313.

8. Imagawa W., Yang J., Guzman C. and Nandi S. 2000. Collagen gel method for the primary culture of mouse mammary epithelium. *In* Ip M. M. and Asch B. B.(eds.) Methods in Mammary Gland Biology and Breast Cancer Research, New York, *Kluwer Academic/Plenum Publishers*, pp.111-123.

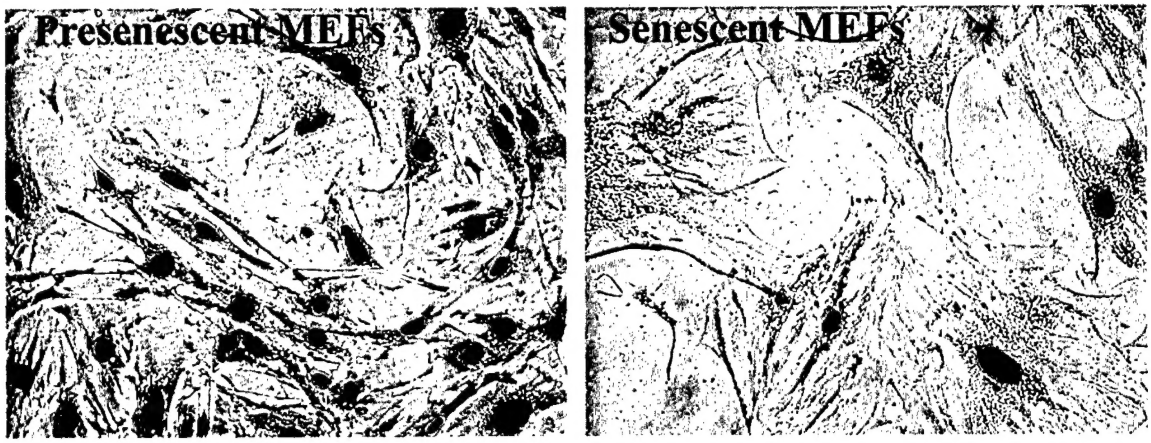
9. Desprez P. Y., Roskelly C., Campisi J. and Bissell M.J. 1993. Isolation of functional cell lines from a mouse mammary epithelial cell strain: the importance of basement membrane and cell-cell interactions. *Mol. Cell. Differen.* 1:99-110.

10. Robles S. J, and Adami G. R. 1998. Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts. *Oncogene* 16:1113-1123.

a


Balb/c MEFs Growth Curve
Log cell #
3
2
1
0
10
20
30
40
Days

b


Presenescent MEFs
Senescent MEFs

c

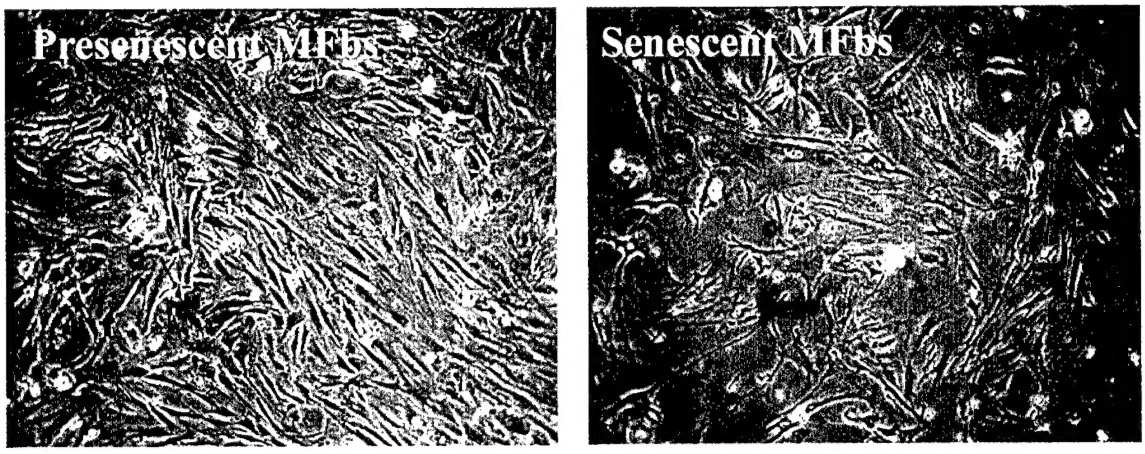

Presenescent MFbs
Senescent MFbs

Figure 1

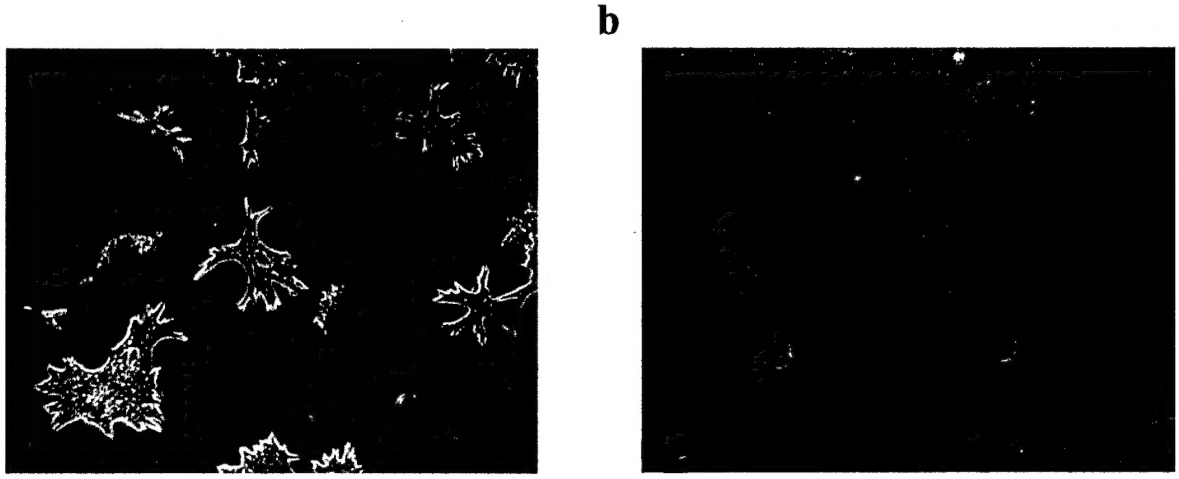


Figure 2

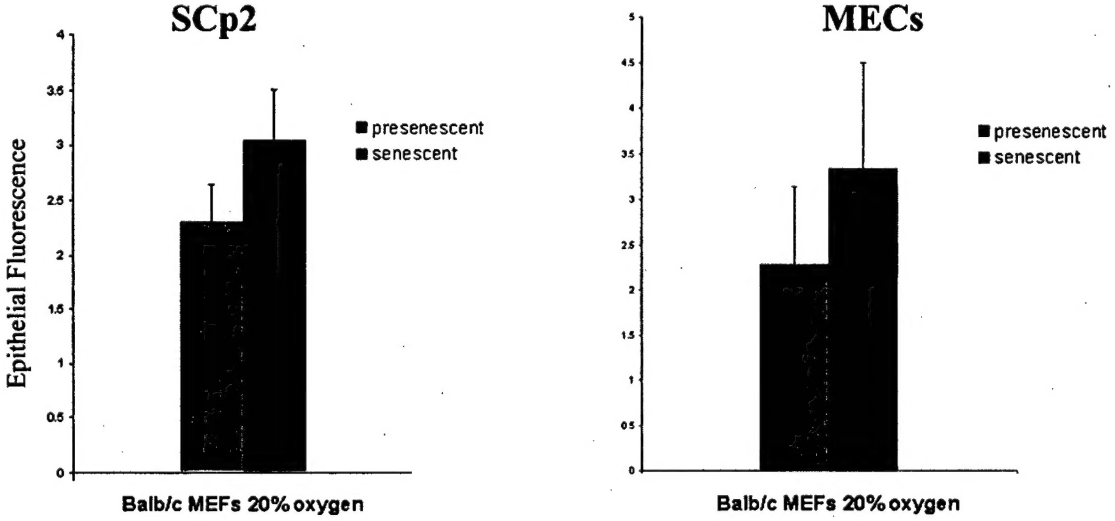


Figure 3

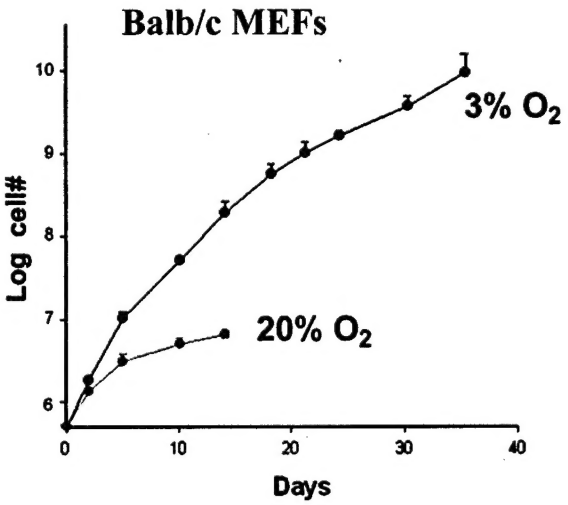


Figure 4